

Effect of DDR Receptors on Cell-Matrix Interaction

Honors Thesis

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Abstract

Mechanical forces exerted by the extracellular matrix (ECM) on cells play an essential role in development, wound healing, and tissue engineering. The ECM in mammalian connective tissue is primarily composed of fibers of collagen type 1. Several collagen binding proteins are known to influence collagen fiber structure and content. How these changes in collagen fiber affect forces exerted by the ECM is not well understood. We recently established that the collagen binding proteins, Discoidin domain receptors (DDR1 and DDR2) alter the native structure and mechanical properties of collagen fibers. The objective of this study was to evaluate how alteration of the ECM environment by DDRs affects mechanical forces exerted on cells. Cell lines stably expressing the extracellular domain (ECD) of DDR1 or DDR2 and DDR2/-KD (DDR2 lacking kinase domain) were used in collagen gel contraction assays. While both DDR2-ECD and DDR2/-KD expressing cells inhibited collagen gel contraction, DDR1-ECD enhanced contraction as compared to controls. To further our understanding of DDR2-ECD affect on cell-matrix interaction we employed DDR2 antibody with the collagen gel contraction assays. DDR2 antibody affects contraction of both nontransfected and transfected cell lines. To confirm that modulation of collagen gel contraction by DDR-ECD expressing cells was due to changes in collagen morphology and not due to changes in the cell cytoskeleton we performed actin staining assays with each cell line with collagen stimulation. Transfected cell lines demonstrated changes in actin organization compared to the nontransfected cell lines. To evaluate the viscoelastic properties of the ECM altered by DDRs, a micro-rheology technique employing optical tweezers was utilized. We demonstrated that the alteration of the ECM by DDRs influences the mechanical forces experienced in cell-matrix interactions.

Introduction

Collagen assembly

Tissue architecture is a critical factor for physiological processes. The extracellular matrix (ECM) in several mammalian connective tissues is the primary structural unit which also forms a scaffold for cells. This matrix is formed from networks of polysaccharides and proteins secreted by cells¹. Collagen is a major fibrous protein of the ECM. . Fibrillar-collagens such as collagen type I are fibril forming collagens. They are secreted as procollagen molecules from connective tissue cells. Once secreted these precursor collagen molecules form collagen fibers through a self-assembly process (collagen fibrillogenesis), which is driven by the intrinsic properties of these molecules². Self-assembled collagen fibrils have been extensively studied and shown to have banded periodic structure⁹. Connective tissue cells can interact with these collagen fibers influencing their size and arrangement through mechanical, chemical, and plasma membrane interaction. Changes in collagen structure can result in architectural changes of the tissue as well as in cell-matrix adhesion and traction forces exerted on the ECM fibers¹. This modified ECM is expected to have major implications in the integrity of tissues and physiological processes.

Several natural agents such as collagen binding proteins influence collagen fibrillogenesis by disturbing the rate of formation and structure of collagen fibers^{5,8}. Soluble collagen-binding proteins such as decorin, fibromodulin, vitronectin as well as cell surface receptors such as integrins interact with collagen type I molecules influencing collagen fibrillogenesis^{5,8}. Ultimately, these changes in collagen arrangement alter the tissue architecture which will affect the mechanical properties of the tissue and in general its physiological (or pathological) role.

Discoidin Domain Receptors

Discoidin domain receptors (DDR) are collagen binding proteins, and are part of the receptor tyrosine kinases family. There are two types of DDRs (DDR1 and DDR2) that are widely expressed in human tissues and provide different functions. DDR1 is expressed in many of our tissues such as skin, brain, and gut. DDR2 has functions in skeletal muscle, heart, and connective tissue³. DDRs are known to regulate many cellular processes such as cell adhesion, proliferation, migration, differentiation, and cell cycle. Structurally, DDRs consist of an extracellular domain (ECD), a transmembrane domain, and an intracellular kinase domain (**figure 1**). Collagen is a ligand for these DDR proteins. Normally, when collagen binds to the DDR ECD, the kinase domain becomes phosphorylated. This phosphorylation causes matrix metalloproteases (MMPs) to become activated, which in turn cleave and degrade collagen in the ECM⁵. DDRs are extensively studied in many malignancies and diseases. DDR1 and DDR2 are highly over-expressed in mammary, ovarian, lung, breast, colon and other various cancers^{3,4}. DDRs are found over-expressed or irregular in diseases such as atherosclerosis, lymphagioleiomyomatosis, osteoarthritis, and rheumatoid arthritis.⁵ Furthermore, DDRs play a significant role in dermal wound healing and can influence the formation of keloid, or scar tissue formation⁶. By studying DDR1 and DDR2 interaction with collagen and their mechanical consequences we can contribute to understanding the role of DDRs role in health and disease.

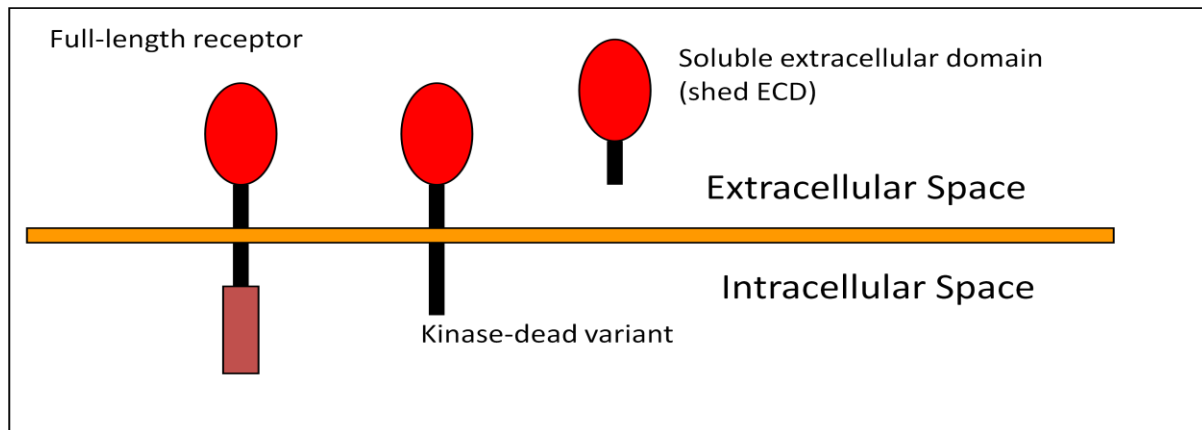


Figure 1: Stably transfected cell lines used in experiments

What is known about DDRs?

The ECD of DDRs is known to be necessary and sufficient for its binding to collagen. Dr. Agarwal's lab has already established that the DDR ECDs affect collagen structure and collagen fibrillogenesis. Recombinant DDR1 and DDR2 ECD proteins were found to delay the formation of collagen fibrils and have an inhibitory affect on collagen fibrillogenesis through the use of turbidity measurements and atomic force microscopy⁷. A later study used Transmission Electron Microscopy (TEM) to show how the cells expressing ECDs of DDR1 or DDR2 **affect** the arrangement of collagen fibers endogenously secreted by the cells⁸. The TEM images in **figure 2** show how DDRs disrupt the periodic banded structure of the collagen fibers⁸. Furthermore, TEM images of cross sections depicted that DDR1 and DDR2 caused a reduction in collagen fiber diameter compared to the control⁸. These studies enable us to conclude that both DDR1 and DDR2 ECDs hinder collagen fibrillogenesis, disrupt rate of fibril formation, and alter collagen fiber structure.

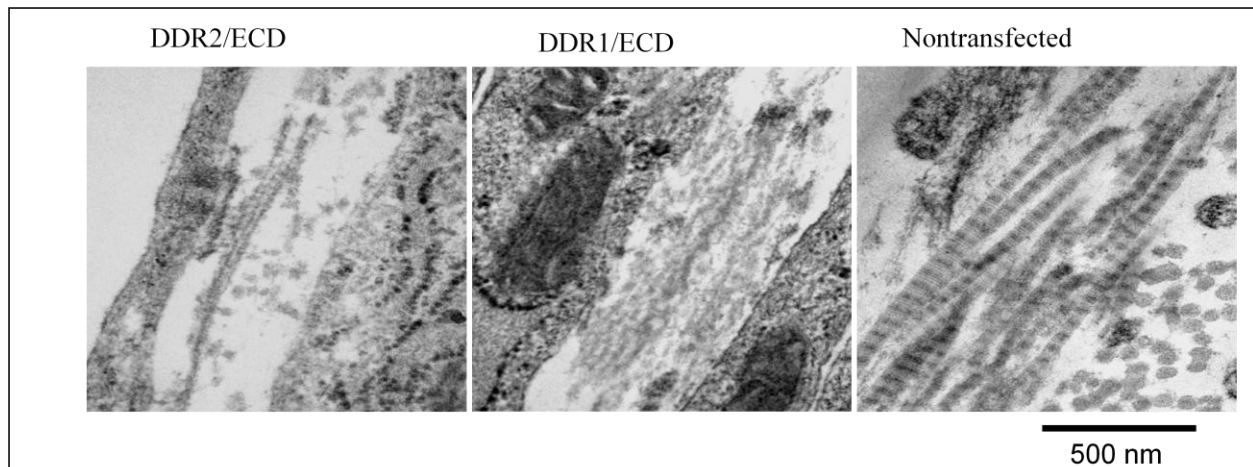


Figure 2: TEM image of collagen fibers secreted by transfected and nontransfected cells^{5,8}

The Agarwal lab has also studied how DDRs affect the mechanical properties of collagen. In another research study TEM images were used to determine the impact of DDR2 on persistence length and the elastic modulus⁹. This report showed that collagen fibers formed in the presence of DDR2 had reduced persistence length and elastic modulus⁹. The DDR2 ECD cell line expresses and secretes the soluble extracellular domain. DDR2/-KD cell line expresses the protein on the cell surface but lacks the kinase domain. Thus DDR ECDS proteins alter both collagen structure and mechanical properties and could potentially impact cell-matrix interaction⁹.

Objectives of this study

The overall objective of this study is to further our understanding of how DDRs influence the ECM by analyzing the effects of DDRs in cell-matrix interaction through various methods.

Objective 1: to determine how changes in the ECM induced by DDR ECDs impact cell-matrix interaction forces

To evaluate DDR ECDs influence on the mechanical forces between the cells and ECM we utilized collagen gel contraction assays. These experiments were conducted and analyzed with

the stable cell lines (DDR1ECD, DDR2ECD, DDR2/-KD) created by the Agarwal lab. Further a DDR2 inhibitor antibody was used in collagen gel assays to evaluate how inhibition of DDR-collagen interaction impacts cell-matrix interaction.

Objective 2: to analyze the effects of DDR ECDs on cell cytoskeleton

To understand if DDR ECDs affect ECM modifications as well as cell cytoskeleton remodeling we employed actin staining assays. Actin staining data was collected for transfected and nontransfected cell lines at various time points after collagen stimulation.

Objective 3: to ascertain how DDR ECDs affect the viscoelastic properties of collagen gels

We utilized optical tweezers to determine the elastic modulus of collagen type 1 gels. In later work, the elastic modulus will be evaluated for collagen gels with and without DDR proteins.

Materials and Methods

Cell culture

To evaluate the morphology of collagen fibers endogenously generated by cells, stably transfected mouse osteoblast cell lines (E13T3) were created to over-express secreted soluble protein DDR1ECD and DDR2ECD or cell surface anchored protein, DDR2/-KD. Western blot analysis of the whole cell lysate and conditioned media confirmed expression of transfected proteins in these cell lines. Nontransfected cell lines were cultured in MEM- α with 10% fetal bovine serum and 1% Antibiotic-Antimycotic (pen-G 10,000 units/ml; streptomycin 10,000 μ g/ml; amphotericin B 25 μ g/ml) from Gibco at 5% CO₂ and at 37°C. Transfected cell lines were cultured in regular 3T3 mouse osteoblast media with 475 μ g/ml of geneticin.

Collagen gel contractions

To evaluate how cells expressing DDR1 ECD, DDR2 ECD, and DDR2/-KD affect mechanical forces in cell-matrix interaction collagen gel contraction assays were employed using the nontransfected 3T3 cell line and stably transfected cell lines. Bovine collagen type I stock solution of 2.1 mg/ml was neutralized to physiological pH with 1M NaOH and brought to a final concentration of 1mg/ml using 3T3 media (MEM- α supplemented with 10% FBS, 1% Penn Strep). The nontransfected and transfected cell lines expressing DDR1 ECD, DDR2 ECD, or DDR2/-KD constructs were each added to the collagen solution at the same cell density. Each well of a 12 well plate contained a final volume of 500 μ L with a final concentration of collagen of 1mg/ml and a cell density of 250000. The samples polymerized for 2 h at 37°C. After two hours of incubation a syringe was used to release the gel around the periphery of each well creating “floating collagen gels.” An additional 500 μ L of media was added to each well. The collagen gels were returned to the 37°C incubator and were imaged digitally every 12 hours for a 48 hour time span. The change in area and perimeter was recorded using ImageJ computer software. The experiment was performed three times and contained three samples of each cell line for each trial.

Collagen gel contractions with antibody

To analyze the affects of DDR2 antibody on collagen gel contractions we employed the same procedure as the collagen gel contraction protocol for the 3T3 nontransfected and DDR2ECD cell lines. However, we prepared samples in triplicates of both cell lines with and without DDR2 antibody (0.03 μ g/mL). After polymerization the collagen gel contractions were again digitally imaged at the 24 hour and 48 hour time point. ImageJ computer software was utilized to measure the change in contractions.

Actin Staining

To analyze the effects of DDRs on the cell cytoskeleton native and the stably transfected cell lines which include: DDR1ECD, DDR2ECD, and DDRKD were cultured on glass coverslips coated with poly-L-lysine. Each coverslip was fixed using 2% formalin, washed, and incubated with phalloidin for actin staining and DAPI for cell nuclei staining. This procedure was repeated for each cell line after exogenously bovine collagen was added. Each cell line was stimulated by collagen for 1 hour, 12 hours, and 24 hours. All samples were imaged and analyzed using fluorescent microscopy.

Collagen gel viscoelastic measurements with optical tweezers

Preparing collagen gel samples for optical tweezers

Collagen gels were similarly prepared to those used in contraction assays. Bovine collagen type 1 stock solution was neutralized with 10M NaOH and brought to a final concentration of 1mg/ml with PBS buffer containing 3 μ m latex beads. 200 μ m of this collagen-bead solution was pipetted into rectangular capillary tubes (Vitrocom, Mountain Lakes, NJ) of inner diameter 0.20 mm, inner width 2 mm, and length 5 cm. After samples were placed in 37°C incubator for 24 hours optical tweezer micro-rheometry was used to obtain measurements.

Optical tweezer measurements

Optical tweezer micro-rheometry was conducted by Prof. Greg Lafyatis and Andrew Morss who are both associated with the Physics Department. Prof. Lafyatis constructed a multi-trap optical tweezer system. Viscoelastic properties of these gels were determined using laser-trap micro-rheometry (**Figure 3**). The laser trap was positioned on one of the microspheres, while it is sinusoidally oscillated. Oscillating frequencies from 0.01 and 1000 Hz and “back focal plane” detection were employed. From the displacement of the microspheres at various frequencies Lafyatis and his lab established a young’s modulus.

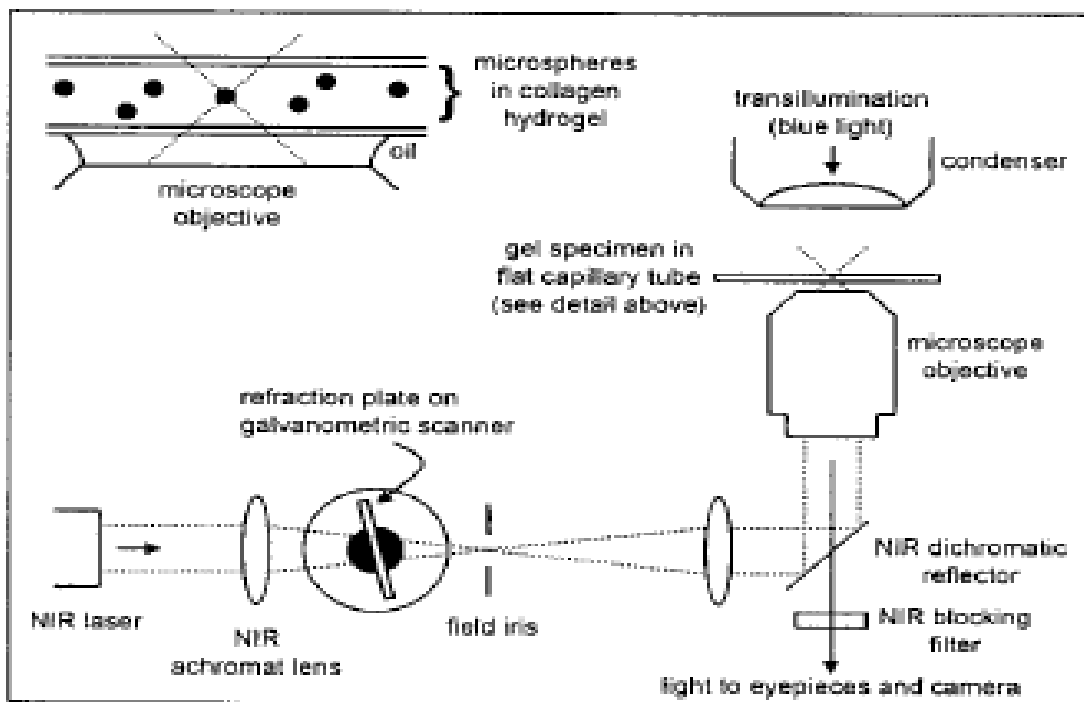


Figure 3: Optical tweezers set up to evaluate elastic modulus of collagen gel containing microspheres⁶

Results

Collagen gel contractions

Collagen gels contract through traction forces exerted on the collagen fibers by the cells. The collagen gel contractions with transfected cell lines were carried out to further our understanding of how the altered ECM affects the cell-matrix interaction and traction forces. The collagen gel contraction assay was performed using the nontransfected cell line and stably transfected cells expressing DDR2 ECD and DDR1ECD, and the membrane anchored protein DDR2 Δ -KD. The expression of each construct was confirmed using Western blotting (**Figure 4**). The digital image in **Figure 5** shows the contraction of each cell line at 24 hours after polymerization. This image shows the contraction of collagen gels is maximal for DDR1ECD expressing cells and minimal for DDR2 ECD expressing cells. DDR2 ECD inhibits collagen gel contraction while

DDR1 ECD enhances as compared to the nontransfected cell line. **Figure 6** quantifies the change in area of these collagen gels at each time point compared to the initial time or when the collagen gels are first released. DDR1 ECD enhances contraction by more than 20% and DDR2 ECD inhibits contraction by more than 20% as compared to the nontransfected. DDR2-/KD demonstrated similar behavior to DDR2ECD but did not inhibit the contraction to the same extent.

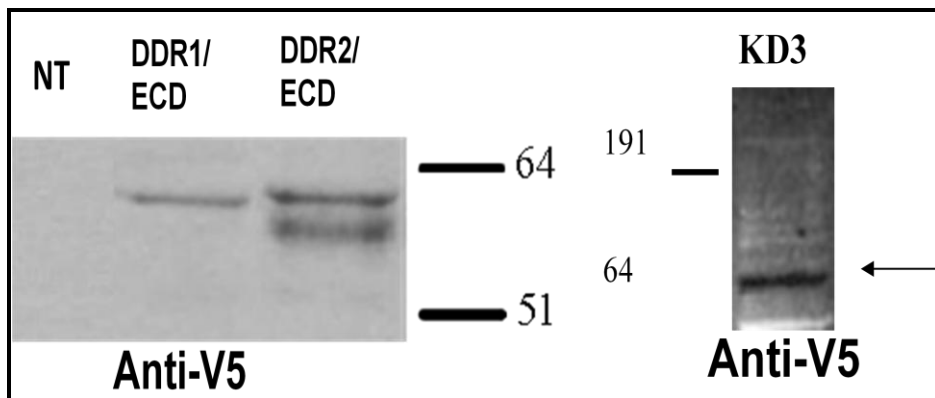


Figure 4: Western blot confirming DDR protein expression of stably transfected cells

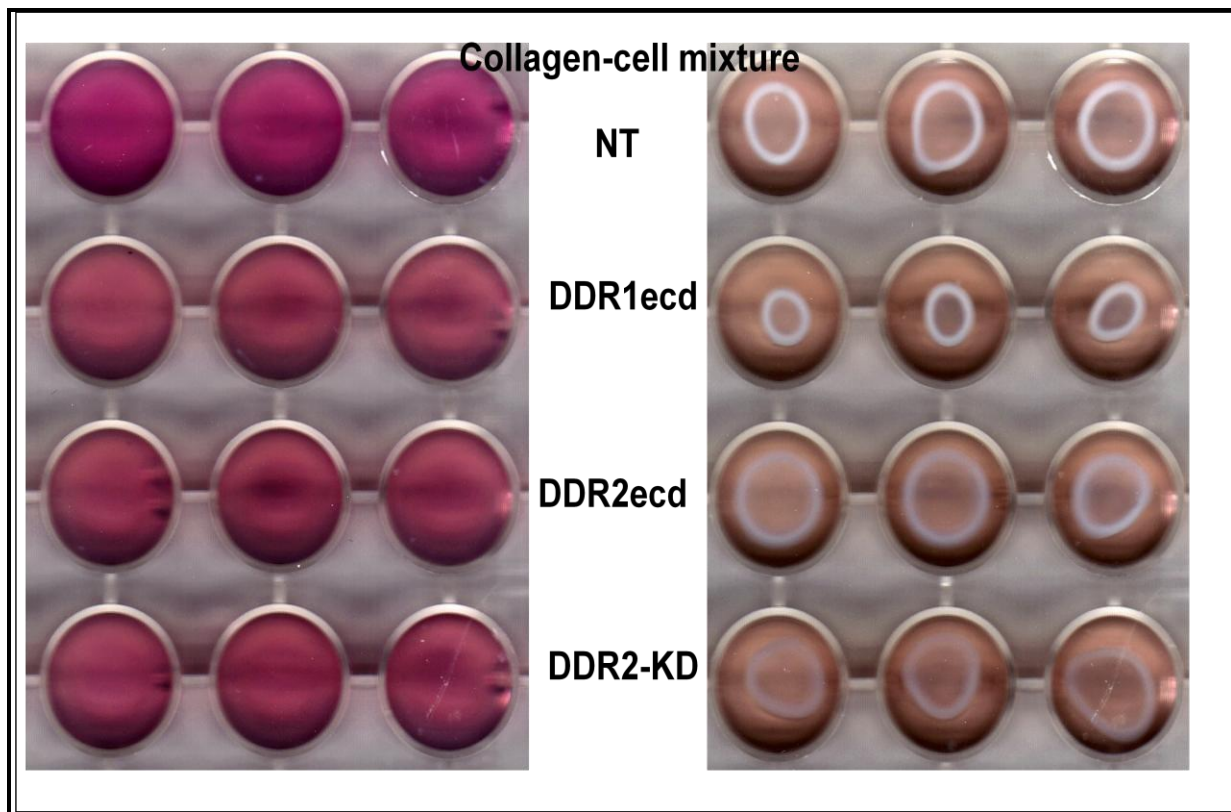


Figure 5: Collagen gel contraction after 24 hours

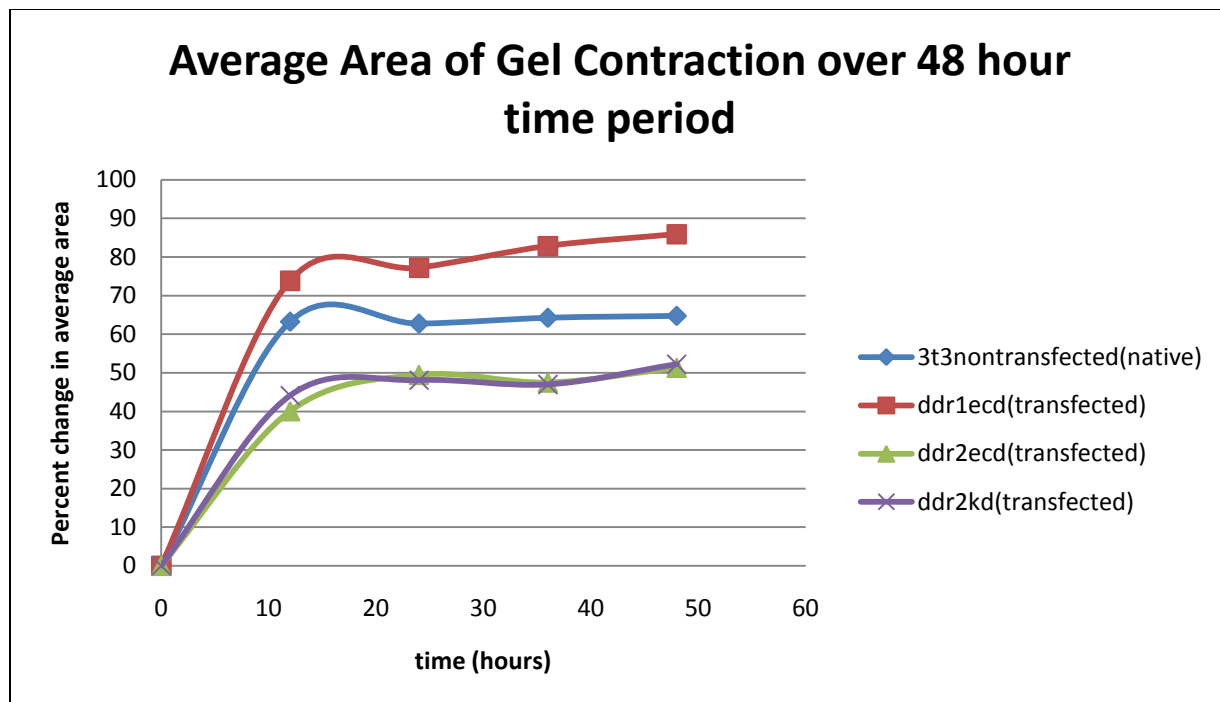


Figure 6: Percent change in area of collagen gels

Collagen gel contractions with DDR2 Antibody

DDR2 antibody was employed to further our understanding of DDR2 ECD on cell-matrix mechanics. This antibody is specific for the DDR2 protein receptor. Collagen gel contractions were analyzed using 3T3 nontransfected and the transfected DDR2 ECD cell lines both with and without DDR2 antibody. The digital images of **Figure 7** demonstrate the effect of the DDR2 antibody on the collagen gel contractions. The image shows a distinct difference in the collagen gels containing DDR2 ECD expressing cells with and without antibody. The image depicts DDR2 ECD without antibody inhibits contraction, while DDR2ECD with antibody significantly enhances contraction. **Figure 8** quantifies the change in area of both the nontransfected and DDR2 ECD collagen gels. The graph shows both nontransfected and DDR2 ECD collagen gel contractions increases with presence of the antibody. However, the antibody affects the DDR2ECD cell line much more, which is shown by the greater increase in contraction of the

collagen gels with and without antibody for the DDR2 ECD (**figure 9**). These results further confirm the role of DDR2 ECD in changing the mechanical forces between the cell and ECM.

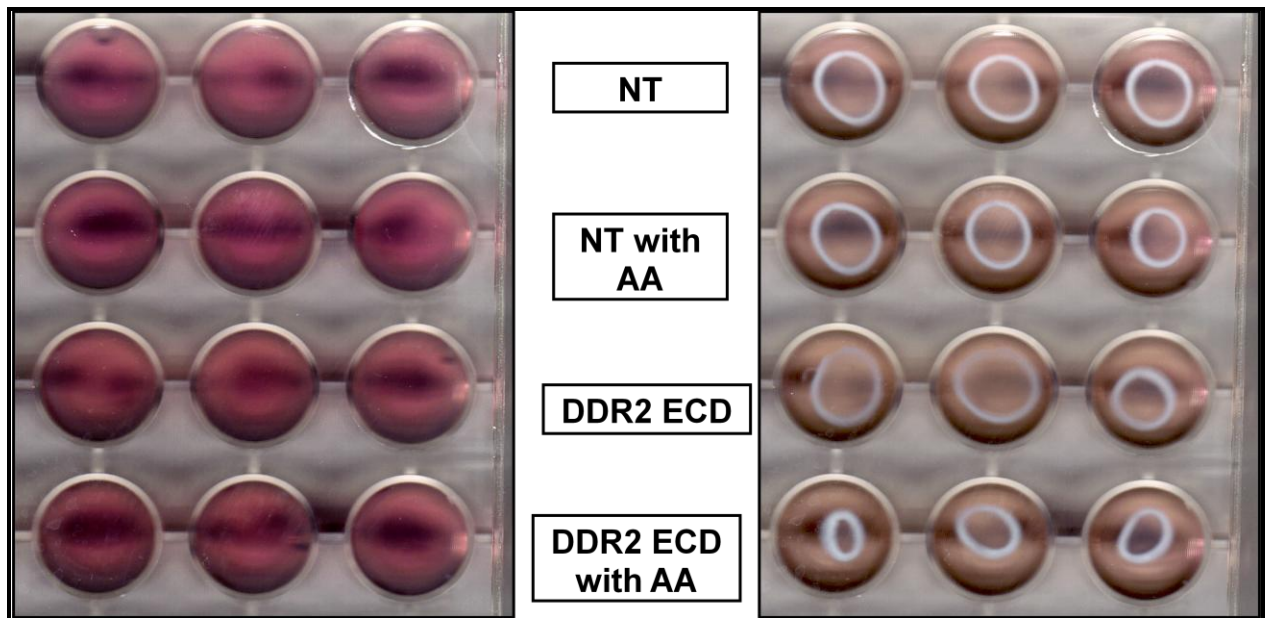


Figure 7: Effect of DDR2 Antibody on collagen gels. Contraction after 48 hours

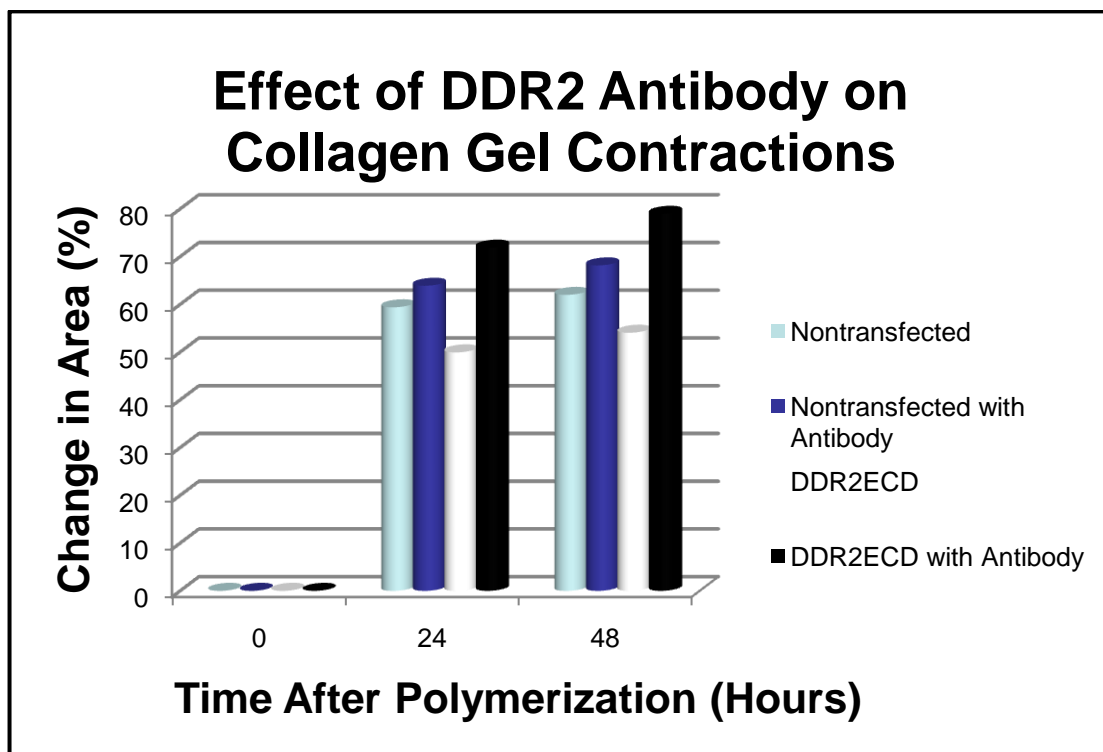


Figure 8: Percent change in area of collagen gels with and without DDR2 Antibody

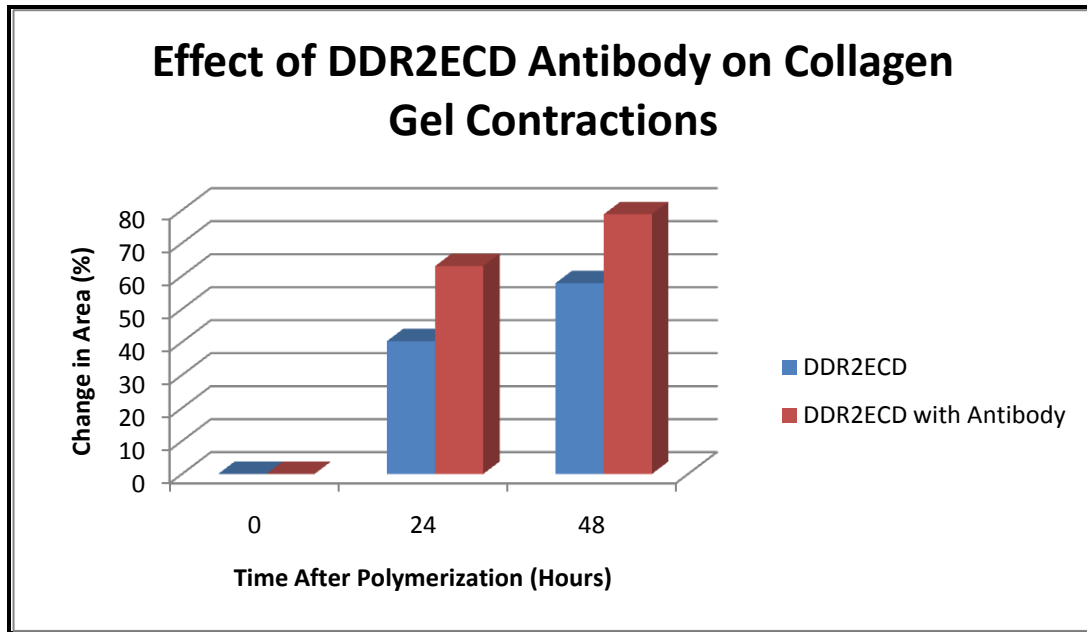


Figure 9: Percent change in area of collagen gels with DDR2 antibody

Actin Staining

The collagen gel contraction results show that DDRs change the mechanical forces between the cell and ECM. To further our understanding of the DDRs role in cell-matrix interaction and if they influence cell stiffness, actin staining experiments for both nontransfected and transfected cell lines were conducted to analyze mechanical changes within the cell. The cell cytoskeleton, specifically actin fibers, plays an essential role in providing the cell mechanical properties. Changes in actin fiber abundance and arrangement will affect the cell's interaction with the ECM and its overall stiffness and mechanical properties. Nontransfected cells and transfected cells including: DDR1ECD, DDR2ECD, and DDR2KD, were stimulated with collagen type 1 for 8 hours. **Figure 10** depicts changes in the actin fibers for the transfected cell lines with collagen stimulation. DDR2 ECD, DDR1ECD, and DDR2 KD all show a reduction in actin fibers and less structured and robust actin network compared to the nontransfected actin staining images before collagen stimulation. However, after 8 hours of collagen stimulation DDR1ECD shows a more abundant and organized actin fiber network similar to the nontransfected image. The collagen stimulation did not affect DDR2ECD and DDR2KD cell lines. The images for these cell lines still depicted a weakened cytoskeleton.

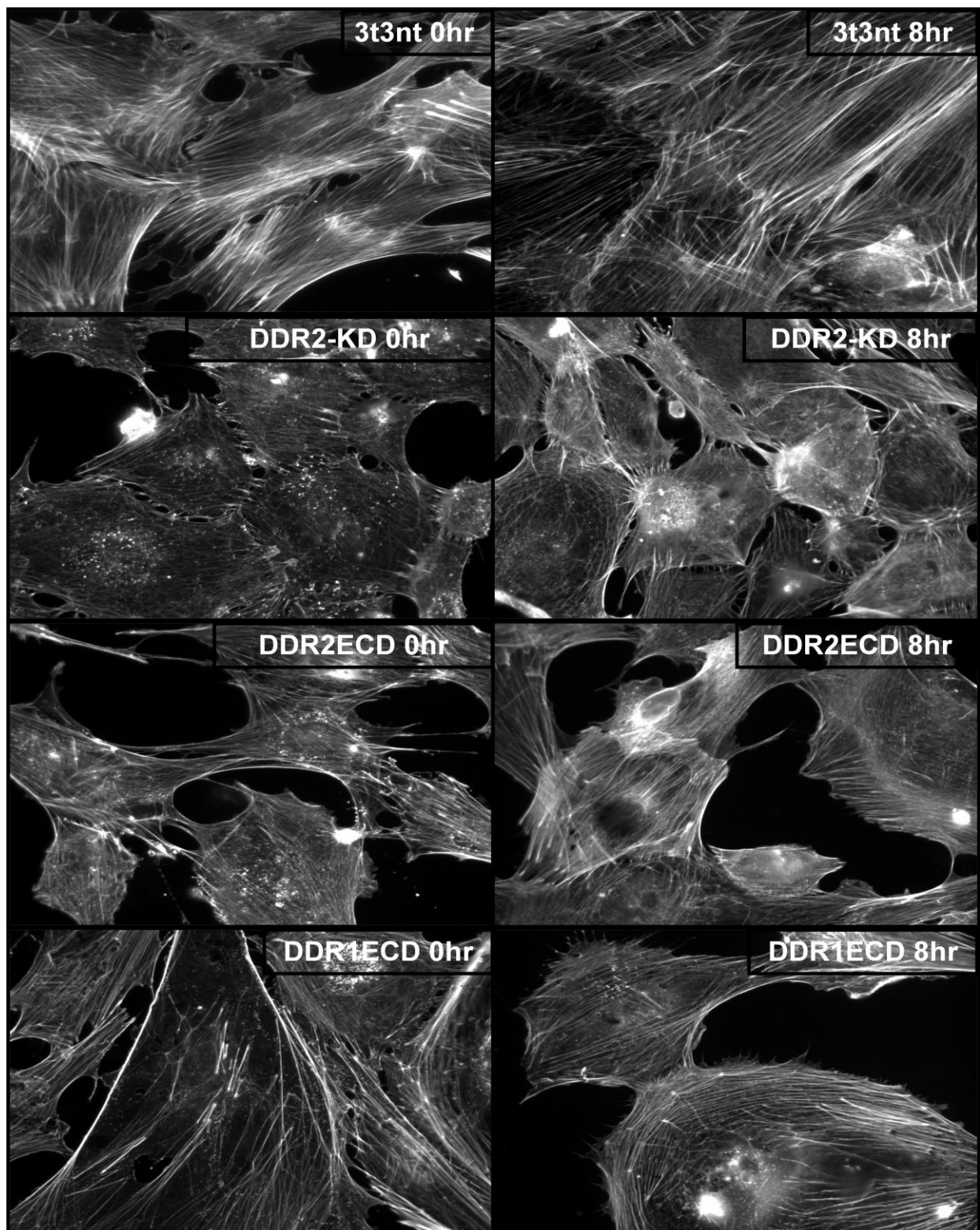


Figure 10: Fluorescence microscopy analysis of actin cytoskeleton before and after incubation with collagen

Optical Tweezers preliminary work

After creating collagen gels containing microspheres in capillary tubes Lafyatis group was able to obtain an elastic modulus for the collagen gel at various points throughout the sample. The table below shows preliminary data of the elastic modulus found at various locations in the collagen gel at two frequencies.

Location	G' (1 Hz) Pa	G' (10 Hz) Pa
2	42	6
3	34	5
4	64	7
5	46	1
6	32	11

Discussion and conclusion

Prior work conducted by Agarwal's lab has confirmed that DDR ECDs do modify the collagen network altering the extracellular matrix. Collagen gel contractions assays were used with DDRs to further understand how this altered extracellular matrix affects the mechanical forces created in cell-matrix interaction. The collagen gel contraction results showed DDR1 ECD and DDR2 ECD had reverse effects on the mechanical properties of these contractions. DDR1 ECD enhanced contraction while DDR2 ECD inhibited contraction as compared to the nontransfected cells. These results confirm DDRs do change the mechanical interaction between the cells and the ECM.

The collagen gel contraction assay was used with nontransfected and DDR2 ECD cell lines in the presence and absence of DDR2 Antibody. The presence of this antibody enhanced gel contractions for both cell lines. However, the presence of DDR2 Antibody affected the contraction with DDR2 ECD cell line to a much greater extent. This binding of DDR2 Antibody to the DDR2 protein on nontransfected and transfected cell lines prevents the DDR2 interaction with ECM resulting in greater contraction and changes in the cell-matrix interaction.

The cell cytoskeleton can also play a role in the mechanical properties between the cell and extracellular matrix. To analyze the effect of DDRs on changes in the cell cytoskeleton actin staining was carried out for both nontransfected and transfected cell lines with collagen stimulations at various time points. Before collagen stimulation transfected cell lines showed a weaker actin network than the nontransfected cells. With collagen stimulation DDR1 ECD and nontransfected cell lines showed a stronger more aligned system of collagen fibers. DDR2 ECD and DDR2 KD cell lines were not affected by the collagen stimulation and maintained a weakened actin network after collagen stimulation. Future work is still needed to better understand and quantify DDR effect on the cell cytoskeleton. This can be accomplished through cell stiffness measurements with Atomic Force Microscopy.

The optical tweezer preliminary data showed the elastic modulus for collagen type 1 gels at various frequencies. This work also needs to be furthered by analyzing the elastic modulus for these collagen gels containing DDR protein to better understand the influence of DDRs on the micro-rheology properties of collagen.

Through this research in this study we found that DDRs affect both the cell cytoskeleton and its ability to exert forces on the matrix as well as the mechanical properties of the matrix itself.

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